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Interactions of the CelS Binding Ligand with Various Receptor Domains of the *Clostridium thermocellum* Cellulosomal Scaffolding Protein, CipA

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The *Clostridium thermocellum* cellulosomal scaffolding protein, CipA, acts as an anchor on the cellulose surface for the various catalytic subunits of the cellulosome, a large extracellular cellulase complex. CipA contains nine repeated domains that serve as receptors for the cellulosomal catalytic subunits, each of which carries a conserved, duplicated ligand sequence (DS). Four representative CipA receptor domains with sequence dissimilarity were cloned and expressed in *Escherichia coli*. The interaction of these cloned receptor domains with the duplicated ligand sequence of CelS (expressed as a thioredoxin fusion protein, TRX-DSCelS), was studied by nondenaturing polyacrylamide gel electrophoresis. TRX-DSCelS formed a stable complex with each of the four receptor domains, indicating that CelS, the most abundant cellulosomal catalytic subunit, binds nonselectively to all of the CipA receptors. Conversely, the duplicated sequence of CipA (in the form of TRX-DSCipA), which is homologous to that of CelS, did not bind to any of the receptors under the experimental conditions.

Clostridium thermocellum degrades crystalline cellulose through the action of an extracellular enzyme complex called the cellulosome (6). The largest component of the cellulosome is a noncatalytic subunit, CipA (formerly CelL or S_L [16, 17]; M_r = 250,000), which consists of nine repeated domains, a cellulose-binding domain (CBD) residing between the second and third repeated domains, and a conserved duplicated sequence (DS) at the COOH terminus (Fig. 1) (2). According to the anchor-enzymes model that we previously proposed (3, 15, 16), CipA binds to cellulose through its CBD and serves as an anchor on the cellulose surface for the catalytic subunits of the cellulosome. These catalytic subunits also contain a conserved DS (ligand) almost exclusively at their COOH termini, which binds to the repeated domains of CipA (receptors) (12). Among these catalytic subunits, most of them β-endoglucanases, CelS stands out as an exoglucanase (4), the most abundant catalytic subunit of the cellulosome (15), and an enzyme essential for degrading crystalline cellulose (17). We have recently demonstrated that CelS forms a stable complex with the third repeated domain of CipA, R3, cloned and expressed in *Escherichia coli* (3). The interaction between CelS and R3 is mediated by the conserved DS of CelS (DSCelS). Binding of CelS to cellulose is dependent on the presence of the CBD in addition to R3. Most importantly, synergism between CelS and CBD-R3 results in enhanced CelS activity toward crystalline cellulose.

Many aspects of the structure-function relationship of the cellulosome are still unknown. In particular, the manner in which the individual subunits are assembled into the cellulosome remains to be elucidated. For example, although the DNA sequences for the nine repeated domains of CipA are fairly conserved, they display different degrees of homology. Whereas five repeats, R3 through R8, show 96 to 100% identity (to R4), R1, R2, and R9 show 69, 81, and 75% identity, respectively. It remained to be demonstrated whether CelS can bind to all of the CipA repeated domains, despite their sequence dissimilarities. In addition, the potential binding of the conserved DS of CipA (DSCipA) to these repeated domains remained to be investigated. In this work, we examined whether DSCelS selectively binds to any of the representative CipA repeated domains individually cloned and expressed in *E. coli*. In addition, we examined whether CipA could bind to itself through interactions between its own DS and receptor domains. The binding was analyzed under nondenaturing conditions, in contrast to the commonly used blotting technique involving denaturing gel electrophoresis.

Expression and purification of the CelS and CipA domains. Cloning of the R3 sequence of CipA by using PCR has been previously described (3). The R1, R2, and R9 sequences of CipA (Fig. 1) were similarly cloned by using *Pfu* (Stratagene, La Jolla, Calif.) or Vent (New England Biolabs, Beverly, Mass.) DNA polymerase. DSCipA and DSCelS were also cloned by using PCR. The primer sets used to generate these clones are summarized in Table 1. The template used for amplifying the R1 and R2 sequences was pJCECL69, which contains the entire *cipA* gene. Plasmid pJCECL69 was constructed by fusing a 1.1-kb fragment containing the 5′ end of the *cipA* gene from pGE1.1 (unpublished results) with the *Eco*RI-*Sma*I fragment from pRG2.0, which contains the 3′ end of the *cipA* gene (2). The template used for amplifying the R9 and DSCipA sequences was pRG2.0 (2), and that used for amplifying the DSCelS sequence was pKK-34 (14). Two plasmid vectors were used for cloning and expressing these PCR products. The R1, R2, and R9 sequences were cloned into pRSETB (Invitrogen, San Diego, Calif.) by using the *Sac*I and *Hin*dIII sites. The DSCipA and DSCelS sequences were cloned into the *Bam*HI and *Sal*I sites of pTRXFUS, a vector designed for generating thioredoxin (TRX) fusion proteins (7). All of the constructs were verified by sequencing the junctions between the insert and the vector.

Expression and purification of R1, R2, and R9 were carried out in a manner similar to that previously described for R3 (3).

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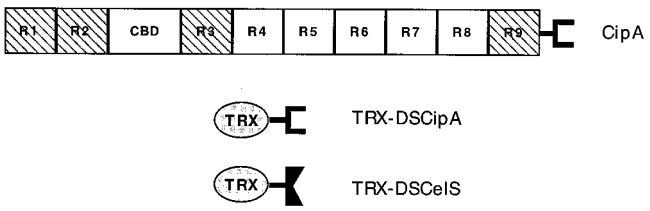

FIG. 1. Schematic representation of the recombinant proteins used in this work. The shaded boxes represent the individually cloned CipA receptor domains. The figure is not drawn to scale.

In brief, the harvested recombinant *E. coli* cells were resuspended in lysis buffer (50 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl, 0.13 mM phenylmethylsulfonyl fluoride) and lysed with lysozyme as described previously (3, 11, 14). After centrifugation at 15,000 × *g* for 10 min, dithiothreitol (DTT; 30 mM) was added to the lysate. The lysate was then heated for 15 min at 75°C (R1 and R2) or 60°C (R9), and the precipitate was removed by centrifugation. It is noteworthy that R2 was stable up to 85°C, while R1 precipitated at this temperature. The R9 supernatant was further purified by immobilized metal affinity chromatography, using a 1-ml HiTrap chelating column (Pharmacia Biotech, Piscataway, N.Y.) equilibrated with 20 mM sodium phosphate (pH 7.3)–500 mM NaCl. The protein was eluted with 100 mM imidazole in 20 mM sodium phosphate (pH 6.1)–500 mM NaCl.

The TRX-DSCelS and TRX-DSCipA gene sequences were expressed in *E. coli* GI698 as described by LaVallie et al. (7). The cells were lysed in lysis buffer, and the supernatant was heat treated at 80°C as described above except that 2.5 mM EDTA was added to the TRX-DSCelS lysate. The TRX-DSCelS supernatant was further purified by anion-exchange chromatography, using a Resource Q column (Pharmacia Biotech) equilibrated with 20 mM Tris-HCl (pH 8.0)–1 mM DTT. The protein was eluted at 0.3 M NaCl.

Protein concentrations were measured by the Bradford method (1), using a Bio-Rad (Hercules, Calif.) protein assay kit with bovine serum albumin as a standard. The purity of the proteins as analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (5) is shown in Fig. 2.

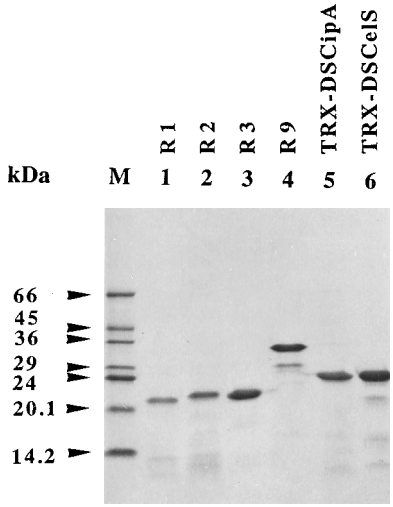

FIG. 2. SDS-polyacrylamide gel (4 to 20%) showing the purity of the recombinant proteins. The proteins were stained with Coomassie blue. Lanes: M, molecular weight markers: 1, R1 (2.0 μg); 2, R2 (2.1 μg); 3, R3 (0.5 μg); 4, R9 (2.9 μg); 5, TRX-DSCipA (2.6 μg); 6, TRX-DSCelS (2.9 μg).

Each of the purified proteins showed a single predominant band in the gel. Their apparent molecular weights were close to their respective calculated molecular weights (Table 1).

Complex formation between TRX-DSCelS and the receptor domains of CipA. Complex formation between TRX-DSCelS and the cloned receptor domains of CipA was carried out by incubating the various recombinant proteins in 50 mM succinate buffer (pH 5.7) containing 10 mM $CaCl_2$ and 5 mM DTT for 10 min at room temperature. Longer incubation times did not increase the degree of complex formation. The resulting complexes were analyzed by nondenaturing PAGE as previously described (3). In every case, the combination of TRX-DSCelS with a receptor domain (R1, R2, R3, or R9) resulted in a new band with a mobility lower than those of the individual proteins (Fig. 3, lanes 3, 5, 7, and 9). This band is presumed to be the complex formed as a result of binding between TRX-DSCelS and the receptor domain. Under the experimental

TABLE 1. PCR primers for cloning the genes encoding DSCelS and various CipA domains of *C. thermocellum* ATCC 27405

Clone	PCR primers[a] (5′→3′)	F or R[b]	Length of the PCR product (bp)	Calculated mol wt of the fusion protein[c]	Apparent mol wt
R1	GCGGAGCTCGGCCACAATGACAGTCGAGA	F			
	GCGCGAAGCTTTTACGGTACGGAACTACCAAGAT	R	436	18,925 (14,998)	22,000
R2	GCGGAGCTCGTCAGACGGTGTGGTAGTAG	F			
	GCGCGAAGCTTTTACGGTGTTGCATTGCCAACGT	R	439	19,367 (15,440)	23,000
R3[d]	GCGGGATCCGGATGATCCGAATGCAATAAAG	F			
	GCGGGTACCTTAATCTCCAACATTTACTCCACCG	R	444	19,440 (15,999)	23,000
R9	GCGCGGAGCTCACTTAAGATAGGCAGAGCAG	F			
	GCGCGAAGCTTTTACATTATTGGAGCCTGTGAAG	R	712	29,011 (25,084)	34,000
DSCelS	GCGGGATCCACCTGGTACTCCTTCTACTA	F			
	GCGGTCGACATTAGTTCTTGTACGGCAATG	R	224	21,202 (8,013)	25,000
DSCipA	GCGGGATCCGACGGGAGATACTTCAGTTTC	F			
	GCGGTCGACTTACTGTGCGTCGTAATCAC	R	246	22,038 (8,849)	25,000

[a] In addition to the sequences flanking the desirable gene segments, the primers incorporated restriction sites compatible with the cloning vector pRSETB or pTRXFUS for in-frame fusion and three to five additional bases at the 5′ ends to facilitate efficient cleavage by restriction enzymes. The *celS* sequence is from reference 13. The *cipA* sequence from reference 2. The DSCelS gene contained sequences corresponding to bases 2003 to 2226 of *celS*, and the R1, R2, R3, R9, and DSCipA genes contained sequences corresponding to bases 2670 to 3105, 3126 to 3564, 4255 to 4698, 7236 to 7947, and 7903 to 8148 of *cipA*, respectively.

[b] F, forward; R, reverse.

[c] Each number in parentheses is the calculated molecular weight of the recombinant protein excluding the fusion sequence.

[d] As previously reported (3).

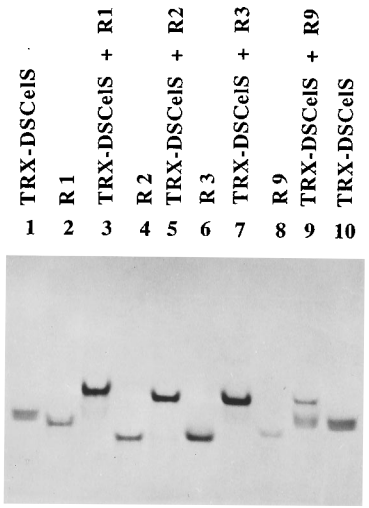

FIG. 3. Complex formation between TRX-DSCelS and R1, R2, R3, or R9 as analyzed by nondenaturing PAGE. Proteins, both individually and in combination, were incubated at room temperature for 10 min in the presence of 10 mM $CaCl_2$ and 5 mM DTT before electrophoresis. The gel (10 to 27%) was prerun for 30 min with 1 mM DTT in the running buffer. The proteins were stained with Coomassie blue. Lanes: 1, TRX-DSCelS (1.9 μg); 2, R1 (2.0 μg); 3, TRX-DSCelS (1.0 μg) plus R1 (2.0 μg); 4, R2 (2.1 μg); 5, TRX-DSCelS (1.0 μg) plus R2 (2.1 μg); 6, R3 (0.3 μg); 7, TRX-DSCelS (1.0 μg) plus R3 (0.3 μg); 8, R9 (2.8 μg); 9, TRX-DSCelS (1.0 μg) plus R9 (2.8 μg); 10, TRX-DSCelS (1.9 μg).

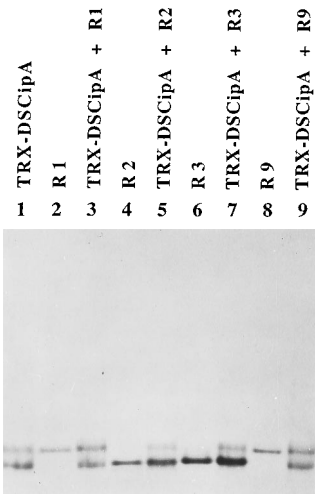

FIG. 4. Nondenaturing PAGE of TRX-DSCipA in combination with R1, R2, R3, or R9. Proteins, both individually and in combination, were incubated at room temperature for 10 min with 10 mM $CaCl_2$ and 5 mM DTT before electrophoresis. The gel (10 to 27%) was prerun for 30 min with 1 mM DTT in the running buffer. The proteins were stained with Coomassie blue. The results show that no complex formation occurred. Lanes: 1, TRX-DSCipA (3.2 μg); 2, R1 (2.0 μg); 3, TRX-DSCipA (3.2 μg) plus R1 (2.0 μg); 4, R2 (2.1 μg); 5, TRX-DSCipA (3.2 μg) plus R2 (2.1 μg); 6, R3 (0.5 μg); 7, TRX-DSCipA (3.2 μg) plus R3 (0.5 μg); 8, R9 (2.6 μg); 9, TRX-DSCipA (3.2 μg) plus R9 (2.6 μg).

conditions used, the equilibrium seemed to favor complex formation, since most of the proteins were seen in their bound form except that in the case of R9, TRX-DSCelS appeared to be in excess (Fig. 3, lane 9). Although TRX-DSCelS showed a single major band in an SDS-gel (Fig. 2, lane 6), two bands of about the same intensity and mobility were observed in the nondenaturing gel (Fig. 3, lanes 1 and 10), suggesting that TRX-DSCelS exists in two different conformations. Upon complex formation, only one conformation seemed to be present.

These results indicate that in addition to binding to R3 as previously reported (3), CelS binds to R1, R2, and R9, despite the sequence differences among these receptors. These results also confirm, as previously reported (3), that DSCelS serves as a binding ligand. The binding of DSCelS to R2 and R3 agrees with the results of Yaron et al. (18), who have reported the binding of various *C. thermocellum* YS cellulosomal subunits, including S8 (CelS [8]), to cohesins 2 and 3 (R2 and R3), as demonstrated by a technique similar to Western blotting (immunoblotting). Our results demonstrate not only that DSCelS binds to the CipA receptor domains but also that the equilibrium favors complex formation. The binding of TRX-DSCelS to R1, R2, and R9 is significant, since the sequences of these receptor domains deviate the most from the other receptor sequences as described above. The differences in the receptor domains apparently have no effect on their abilities to bind to DSCelS. These results therefore indicate that although subtle differences in affinity may exist, CelS binds nonselectively to and forms a stable complex with any of the nine receptor domains of CipA.

Analysis of complex formation between TRX-DSCipA and the CipA receptor domains. DSCipA shows about 42% similarity (23% identity) with DSCelS. It was of interest, therefore, to examine whether DSCipA could bind to any of the receptor domains. Nondenaturing gel analysis of complex formation between TRX-DSCipA and the various CipA repeated domains was carried out as described above. The results indicate that no complexes are formed, since no new bands were seen when TRX-DSCipA was combined with any of the receptor proteins (Fig. 4). As with TRX-DSCelS, TRX-DSCipA appeared as two bands on the nondenaturing gel (Fig. 4, lane 1), even though it appeared as a single band on the denaturing gel (Fig. 2, lane 5). This result again indicates the possible existence of two different conformations of this protein. Using a blotting technique after denaturing gel electrophoresis, Salamitou et al. (10) found that DSCipA bound to a different set of *C. thermocellum* proteins than did DSCelD, another catalytic subunit, but not to CipA itself. Our results show that even under nondenaturing conditions, TRX-DSCipA does not bind to the receptor domains. Thus, despite the similarity between DSCipA and DSCelS, these two ligands apparently have distinct binding specificities. In opposition to our previous hypothesis (15), CipA does not seem to bind to itself through an interaction between its DS and receptor domains, at least not under the experimental conditions used. The function of DSCipA remains to be elucidated.

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